

Published in final edited form as:

Biochem Biophys Res Commun. 2010 July 30; 398(3): 513–518. doi:10.1016/j.bbrc.2010.06.110.

Establishment of sandwich ELISA for soluble alpha-Klotho measurement: Age-dependent change of soluble alpha-Klotho levels in healthy subjects

Yuji Yamazaki¹, Akihiro Imura^{2,9}, Itaru Urakawa³, Takashi Shimada³, Junko Murakami³, Yukiko Aono³, Hisashi Hasegawa³, Takeyoshi Yamashita³, Kimihiko Nakatani⁴, Yoshihiko Saito⁴, Nozomi Okamoto⁵, Norio Kurumatani⁵, Noriyuki Namba⁶, Taichi Kitaoka⁶, Keiichi Ozono⁶, Tomoyuki Sakai⁷, Hiroshi Hataya⁷, Shoji Ichikawa⁸, Erik A. Imel⁸, Michael J. Econs⁸, and Yo-ichi Nabeshima^{2,9}

¹Antibody Research Laboratories, Kyowa Hakko Kirin Co. Ltd., Tokyo, 194-8533, Japan

³Innovative Drug Research Laboratories, Kyowa Hakko Kirin Co. Ltd., Tokyo, 194-8533, Japan

²Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, 606-8502, Japan

⁴First department of Internal Medicine, Nara Medical University, Nara, 634-8521, Japan

⁵Department of Community Health and Epidemiology, Nara Medical University, Nara, 634-8521, Japan

⁶Department of Pediatrics, Osaka University Graduate School of Medicine, Osaka 525-0871, Japan

⁷Department of Pediatric Nephrology, Tokyo Metropolitan Children's Medical Center, Tokyo, 204-8567, Japan

⁸Division of Endocrinology, Indiana University School of Medicine, Indiana, 46202-5135, USA

⁹Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Saitama, 332-0012, Japan

Abstract

Background— α -Klotho (α Kl) regulates mineral metabolism such as calcium ion (Ca^{2+}) and inorganic phosphate (Pi) in circulation. Defects in mice result in clinical features resembling disorders found in human aging. Although the importance of transmembrane-type α Kl has been

Correspondence and requests for reprint should be addressed to Yo-ichi Nabeshima, M.D. & Ph.D. nabemr@lmls.med.kyoto-u.ac.jp Phone: +81-75-753-4422, FAX: +81-75-753-4686 Yuji Yamazaki, M.S. yuji.yamazaki@kyowa-kirin.co.jp Phone: +81-42-725-2555, FAX: +81-42-726-8330.

Each author's: Yuji Yamazaki: yuji.yamazaki@kyowa-kirin.co.jp Akihiro Imura: imura@lmls.med.kyoto-u.ac.jp Itaru Urakawa: itaru.urakawa@kyowa-kirin.co.jp Takashi Shimada: takashi.shimada@kyowa-kirin.co.jp Junko Murakami: junko.murakami@kyowa-kirin.co.jp Yukiko Aono: yukiko.aono@kyowa-kirin.co.jp Hisashi Hasegawa: hisashi.hasegawa@kyowa-kirin.co.jp Takeyoshi Yamashita: takeyoshi.yamashita@kyowa-kirin.co.jp Kimihiko Nakatani: nkimihik@nmu-gw.narmed-u.ac.jp Yoshihiko Saito: yssaito@narmed-u.ac.jp Nozomi Okamoto: onozomi@narmed-u.ac.jp Norio Kurumatani: knorio@narmed-u.ac.jp Noriyuki Namba: nnamba@ped.med.osaka-u.ac.jp Taichi Kitaoka: tkitaoka@ped.med.osaka-u.ac.jp Keiichi Ozono: keioz@ped.med.osaka-u.ac.jp Tomoyuki Sakai: tomoyuki_sakai@tmhp.jp Hiroshi Hataya: hiroshi_hataya@tmhp.jp Shoji Ichikawa: ichikawa@iupui.edu Erik A. Imel: eimel@iupui.edu Michael J. Econs: mecons@iupui.edu Yo-ichi Nabeshima: nabemr@lmls.med.kyoto-u.ac.jp

demonstrated, less is known regarding the physiological importance of soluble-type α Kl (s α Kl) in circulation.

Objectives—The aims of this study were: 1) to establish a sandwich ELISA system enabling detection of circulating serum s α Kl, and 2) to determine reference values for s α Kl serum levels and relationship to indices of renal function, mineral metabolism, age and sex in healthy subjects.

Results—We successively developed an ELISA to measure serum s α Kl in healthy volunteers (n=142, males 66) of ages (61.1 ± 18.5 yr). The levels (mean \pm SD) in these healthy control adults were as follows: total calcium (Ca; 9.46 ± 0.41 mg/dL), Pi (3.63 ± 0.51 mg/dL), Blood urea nitrogen (BUN; 15.7 ± 4.3 mg/dL), creatinine (Cre; 0.69 ± 0.14 mg/dL), 1,25 dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$; 54.8 ± 17.7 pg/mL), intact parathyroid hormone (iPTH; 49.2 ± 20.6 pg/mL), calcitonin (26.0 ± 12.3 pg/mL) and intact Fibroblast growth factor (FGF23; 43.8 ± 17.6 pg/mL).

Serum levels of s α Kl ranged from 239 to 1266 pg/mL (mean \pm SD; 562 ± 146 pg/mL) in normal adults. Although s α Kl levels were not modified by gender or indices of mineral metabolism, s α Kl levels were inversely related to Cre and age. However, s α Kl levels in normal children (n=39, males 23, mean \pm SD; 7.1 ± 4.8 years) were significantly higher (mean \pm SD; 952 ± 282 pg/mL) than those in adults (mean \pm SD; 562 ± 146 , $P < 0.001$). A multivariate linear regression analysis including children and adults in this study demonstrated that s α Kl correlated negatively with age and Ca, and positively with Pi. Finally, we measured a serum s α Kl from a patient with severe tumoral calcinosis derived from a homozygous missense mutation of α -klotho gene. In this patient, s α Kl level was notably lower than those of age matched controls.

Conclusion—We established a detection system to measure human serum s α Kl for the first time. Age, Ca and Pi seem to influence serum s α Kl levels in a normal population. This detection system should be an excellent tool for investigating s α Kl functions in mineral metabolism.

Keywords

alpha-Klotho; ELISA; calcium; phosphate; tumoral calcinosis

Introduction

α -kl gene encodes a type I membrane protein with expression restricted to parathyroid glands, the choroid plexus and the kidney [1-4]. α Kl binds to Na^+ , K^+ -ATPase to regulate PTH secretion and is involved in transepithelial calcium concentration. In response to altered extra-cellular calcium concentrations, α Kl is rapidly translocated from endosomal organella to the plasma membrane together with Na^+ , K^+ -ATPase and simultaneously the extracellular domain of α Kl is cleaved and secreted into the blood circulation and cerebrospinal fluid (CSF) [5,6]. The increased Na^+ gradient produced by elevated Na^+ , K^+ -ATPase activity drives PTH secretion in parathyroid glands and transepithelial transport of calcium in the kidney and choroid plexus [5]. Accordingly, it is assumed that α Kl levels in the serum and CSF mirror the molecular actions of the cellular form of α Kl in these tissues.

α Kl also binds to fibroblast growth factor 23 (FGF23), which was discovered in studies of autosomal dominant hypophosphatemic rickets (ADHR) [7] and later tumor-induced osteomalacia (TIO) [8, 9]. FGF23 (i) is produced and secreted from bone in response to

serum levels of phosphorus and 1,25(OH)₂D [10-12], (ii) binds to FGF receptor1 (FGFR1), both suppressing 1 α -hydroxylase (CYP27B1) expression and stimulating 24-hydroxylase (CYP24A1) expression in kidney [13,14], and (iii) downregulates protein amounts of Na⁺ dependent-phosphate transporter (NaPi) IIa/c to the brush border membrane of proximal tubules thus decreasing phosphate reabsorption [11].

α Kl contributes to integrate mineral homeostasis. Consequently, disturbances of α Kl expression impair mineral metabolism via multiple mechanisms involving FGF23 signaling [13, 14], PTH secretion and transepithelial calcium transport. Recently, a patient with autosomal recessive hyperphosphatemic tumoral calcinosis shed new light on the impact of α Kl [15]. Mutation analysis revealed a missense mutation in α Kl, and in vitro studies indicated that α Kl translocation to the plasma membrane was impaired [16]. Therefore, analysis of serum α Kl levels may lead to greater understanding of disorders of mineral homeostasis.

In the present study, we developed an ELISA system to measure circulating s α Kl concentrations in serum from human subjects for the first time. We further analyzed and compared s α Kl levels of both healthy volunteers and a case with the *α -klotho* gene mutation [16]. Finally, we discuss the potential utility in measuring serum α Kl in clinical disorders.

Materials and methods

Plasmid construction

Human full length α -Klotho (fl- α Kl; 1012 amino acids(a.a), RefSeq ID: NP_004786)-cDNA and cDNA encoding extracellular domain of α -Klotho (s α Kl; 1-979a.a.) were amplified from total human kidney cDNAs by PCR and consequently cloned into pLP-CMVneo and pLP-IRESneo, respectively, by In fusion PCR kit (Clontech).

Cell culture

pLP-CMVneo-fl- α Kl was transfected into HEK293 cells by the calcium-phosphate method. pLP-IRESneo-s α Kl was transfected into CHO cells, by the Lipofectamine method (Invitrogen). Then, cells stably expressing either fl- α Kl or s α Kl were selected by G418 and cloned by limiting dilution. HEK293 cells expressing fl- α Kl were grown in DMEM supplemented with 10% Fetal Calf Serum (FCS) and 1mg/mL of G418. CHO cells expressing s α Kl were grown in MEM α supplemented with 10% FCS and 2mg /mL of G418.

s α Kl protein purification

Recombinant s α Kl protein was purified from serum-free conditioned media (SFMII, Gibco) of CHO cells stably expressing s α Kl by a series of chromatographic steps employing Q Sepharose XL, Butyl Sepharose FF, Heparin Sepharose HP, Q Sepharose HP, SP Sepharose HP, DEAE Sepharose FF, Phenyl Sepharose HP, Q Sepharose HP, Lentil Lectin sepharose 4B, and DEAE Sepharose FF (All sepharoses were purchased from GE healthcare). The concentration of purified s α Kl protein was determined by absorbance at 280 nm.

Animals

All mice were purchased from Charlesriver Inc. Japan and were maintained in SPF conditions according to the institutional guidelines of Kyowa Hakko Kirin Co., Ltd.

Antibodies

Monoclonal anti-human α -Klotho antibodies were generated by immunizing B6D2F1 mice once a week for 5 weeks with pLP-CMVneo-fl- α Kl vector by TransIT in vivo gene delivery system (Takara Bio). Hybridomas were obtained by fusing spleen cells and Sp2/o mouse myeloma cells (ATCC ID CRL1581) with polyethylene glycol. Then, hybridomas producing anti- α -Klotho antibody were selected by cell ELISA and flow cytometry analysis by using HEK293 cells stably expressing fl- α Kl. Finally, nine monoclonal antibodies were purified with Protein G Sepharose 4FF (GE healthcare) and we next tested all pairs of antibodies with high affinity against recombinant s α Kl for sandwich ELISA by using conjugated antibodies with biotin as detection antibodies. A pair of antibodies that have the highest reactivity to s α Kl protein were named 67G3 (IgG1, kappa) as a capture antibody and 91F1 (IgG1, kappa) as a detection antibody, respectively. Rabbit anti-human α -Klotho polyclonal antibodies (N116 and C939) were generated by immunization of two different human α -Klotho partial polypeptides from N-terminal region (PLQPATGDVASDSYNNVFRDT, 116-136a.a.) and C-terminal region (ASMKHYRKIIDSNGFPGPETLERFC, 939-963a.a.), respectively.

Subjects

Healthy Asian volunteers (n=181, 89 males) between 0.1 and 88 years of age participated in our study. Subjects had no known medical conditions. A patient with a missense mutation of the *α -klotho* gene was described in a previous paper [16]. The study protocol was approved by the ethics committee on human research at Kyoto University Graduate School of Medicine, Tokyo Metropolitan Children's Medical Center, Osaka University Hospital, Nara Medical University Hospital and Indiana University. Written informed consent was obtained from subjects prior to enrollment. All subjects had normal serum Ca, Pi, 1,25(OH)₂D, iPTH, calcitonin, FGF23 levels and normal renal function as assessed by BUN and serum Cre levels.

Measurement of serum parameters

Blood samples were drawn from a forearm vein in the morning after overnight fasting. Sera were obtained by centrifugation and immediately stored at -30°C. Serum FGF23 concentrations were measured by a FGF23 kit (Kainos) which can only measure the intact FGF23 [9]. Ca, Pi, BUN, Cre and Alb were measured at SRL Inc. Japan using standard clinical methods. Serum levels of iPTH, calcitonin and 1,25(OH)₂D were measured only in adults (>20 years) at SRL Inc. because of limited serum quantity in children. The mean values of age and serum biochemical tests from each group are shown in Table 1.

Immunoprecipitation and Western blot

500 μ L of sera from two healthy subjects and purified s α Kl protein were incubated with resin (NHS-activated Sepharose 4 FF; GE healthcare) conjugated with 67G3, 91F1 or isotype-

matched control antibody (mouse IgG1) at 4°C for 2h. The specifically retained proteins on the resin were solubilized by boiling in SDS-PAGE sample buffer with the reducing agent and separated by SDS-PAGE. Following electrophoresis, proteins were transferred to PVDF membrane and bound with the biotin labeled N116 or C939 antibodies followed by HRP-conjugated streptavidin (DAKO). Protein bands were visualized by the ECL system (GE healthcare)

ELISA procedure

Wells of microtiter plates were coated with 1 µg/mL of 67G3 monoclonal antibody in 100 µL of carbonate buffer (pH9.5) for 18h at 4 °C and then blocked with 1% BSA in PBS for 1h at 37°C. Samples (50µL) were premixed with dilution buffer (50µL; PBS, pH7.4, containing 0.05% Tween20, 1% BSA, and 50µg /mL purified normal mouse IgG). The mixtures were loaded and incubated for 1h at room temperature (RT), followed by the addition of 100µL 91F1-Fab' conjugated with HRP by using N-(6-Maleimidocaproyloxy)succinimide (EMCS, Dojindo) in PBS for 30 min at RT, and the reaction was visualized by the addition of 100µL of chromogenic substrate (TMB+, Dako) for 30 min at RT. The reaction was stopped with 100µL of 1N H₂SO₄ and absorbance at 450nm was measured with a reduction at 570 nm using ELISA plate reader. Plates were washed 6 times with washing buffer (PBS, pH7.4, containing 0.1% (v/v) Tween 20) after each step. A standard curve was established by a serial dilution of recombinant sαKl.

Results

Establishment of anti-αKl mouse monoclonal antibodies for sandwich ELISA

To establish mouse monoclonal antibodies with strong affinity for human αKl protein, we immunized mice with a human full-length αKl expressing vector and screened the resulting hybridomas by measuring the binding affinities to human αKl expressing cells. Nine antibodies which showed high and specific affinities to an extracellular domain of human αKl protein were tested for compatibility as a capture antibody or detection antibody in sandwich ELISA to detect sαKl protein. We finally selected a pair of antibodies (67G3 for a capture antibody and 91F1 for a detection antibody). While these antibodies are not useful for Western blot assays, both antibodies have a high affinity in immunoprecipitation to sαKl protein as well as in Cell ELISA and Flow cytometry to a transmembrane type of αKl (data not shown), indicating that both antibodies specifically recognize a tertiary protein structure of an extracellular domain of αKl.

Sandwich ELISA for the detection of sαKl in human sera

A typical standard curve is shown in Fig.1A of the purified recombinant human sαKl protein concentration between 94 pg/mL and 6000 pg/mL. The intra- and inter-assay coefficients of variation (CV) were calculated by measuring three different doses with purified human sαKl protein. As shown in Table S1A and S1B, both CVs ranged from 2.7 to 9.8%. The accuracy of this assay in the presence of human serum and plasma was determined by serially diluting recombinant human sαKl in 50% human serum and plasma. The measured sαKl levels were then compared with theoretical sαKl levels contributed by the addition of recombinant sαKl and the endogenous sαKl in serum and plasma. As shown in Table S1C, measured sαKl

levels were 92.6% (in serum) and 94.1% (in plasma) of expected values. Furthermore, the serum dose-response curves were parallel to the purified recombinant sKl dose-response curves (data not shown). Moreover, addition of recombinant human purified FGF23 (1 μ g/mL) did not affect a measurement (data not shown). Serum samples subjected to quick freezing at -80°C and thawing at RT did not show a significant decrease during three cycles (data not shown). These results indicate that sKl can be measured with acceptable accuracy in the presence of 50% human serum or plasma.

Immunoprecipitation of plasma sKl protein from healthy subjects

To address whether both antibodies bind to the endogenous sKl in human plasma as well as the recombinant extracellular (soluble) α Kl, immunoprecipitation was performed. Both 67G3 and 91F1 antibodies immunoprecipitated sKl from plasma from healthy adult subjects (Fig.1B) as well as the recombinant human sKl (rhsKl). Furthermore, the densities of the immunopositive bands for precipitated sKl (about 130kDa) precisely corresponded to the concentrations of sKl represented by ELISA (rhsKl: 5000 pg/mL, plasma 1: 575 pg/mL and plasma 2: 1272 pg/mL). These data suggest that both capture and detection antibodies can bind to endogenous sKl in blood, indicating that our sandwich ELISA system can specifically detect and accurately measure the circulating sKl.

Circulating sKl concentrations in healthy adults

In order to determine the reference range of serum sKl and to address the correlation of several mineral parameters with sKl in healthy adults, we next analyzed serum levels of sKl and indices of mineral metabolism in 142 healthy adults (age 20 years old or over) (Table 1). The serum sKl concentrations of healthy adults ranged from 239 to 1266 pg/mL (mean \pm SD; 562 ± 146 pg/mL). There was no apparent correlation of sKl levels with gender, iPTH, 1,25(OH) $_2$ D, calcitonin, Ca, Pi, BUN, Cre or FGF23 by simple regression analyses. However, sKl levels were slightly, but significantly correlated with age ($r=-0.199$, $P=0.017$) and Cre ($r=-0.183$, $P=0.030$) by simple regression analyses.

Circulating sKl concentrations in healthy children

As mineral metabolism dramatically changes in childhood, we compared concentrations of sKl between adults and children (<17 years) (Table 1). As expected, all factors measured in this study (Ca, Pi, Cre, BUN and FGF23) were significantly different between adults and children. The serum sKl concentrations of normal children were significantly higher than those of adults (mean \pm SD; 952 ± 282 pg/mL in children vs 562 ± 146 pg/mL in adults, student's t-test, $P<0.001$, Fig.2A), indicating that serum sKl correlated with age. The extent of correlation of sKl with other serum factors examined by simple regression analysis was shown in Fig.2B-F. Scl levels were found to correlate considerably with Pi (Fig.2C $r=0.517$, $P<0.001$), Cre (Fig.2E, $r=-0.538$, $P<0.001$) and FGF23 (Fig.2F $r=-0.350$, $P<0.001$) concentrations as well as age (Fig.2B, $r=-0.599$, $P<0.001$). In addition, sKl levels also slightly, but significantly, correlated with BUN (Fig.2D, $r=-0.203$, $P=0.006$) but sKl levels did not significantly correlate with calcium.

Next, factors independently associated with serum sKl levels were examined using a multiple regression model. Serum sKl exhibited significant and independent associations

with age ($P<0.001$), Ca ($P=0.004$) and Pi ($P=0.001$), but no significant associations with BUN ($P=0.411$), Cre ($P=0.051$), or FGF23 ($P=0.089$) as shown in Table S2. Pi was positively correlated with sKl. Interestingly, Ca and age were negatively correlated with sKl in this model, while Ca was a not significant factor with sKl by simple regression analysis.

Examination of sKl levels in a patient with a α Kl mutation

Ichikawa et al. have recently reported a case of hyperphosphatemic tumoral calcinosis with hypervitaminosis D due to missense mutations in human *α -klotho* gene (a single amino acid substitution from histidine¹⁹³ to arginine). The authors concluded that, at least in part, disruption of the architecture of α Kl protein results in a failure to translocate to the plasma membrane [16]. Consequently the subject's sKl level was expected to be low. To test this prediction, we measured serum from the patient and found that the serum sKl level was 337 pg/mL. Because the patient was 14 years old, we prepared serum from age-matched controls ($n=11$, mean \pm SD: 13.5 ± 1.7 years from 11.2 to 16.6 years old). As shown in Fig. 2G, sKl levels of age-matched controls ranged from 652 to 1146 pg/mL, and these values all fit within the mean value \pm 2SD. As anticipated, the subject with α -Klotho mutation had low concentrations of sKl compared with age-matched controls.

Discussion

We developed an ELISA to successfully measure sKl in human subjects and demonstrated positive relationships with phosphorus, and negative relationships with age and Ca^{2+} in multiple linear regression analysis. Since low calcium stimuli induce α Kl secretion [5], the negative relationship appears compatible. We have previously proposed a novel negative feedback system involving FGF23 and α Kl for maintenance of normal phosphate and $1,25(\text{OH})_2\text{D}$ levels [16,13]. Based on the fact that FGF23 is a negative regulator of phosphate concentration (high FGF23 suppresses phosphate reabsorption in kidney), FGF23 correlates with phosphate in a positive manner (high phosphate induces high FGF23 level). Predictably, sKl correlates negatively with FGF23, but positively with phosphate in simple regression analysis. We have proposed a mineral homeostatic system integrated by α Kl, where Ca^{2+} and PTH levels are acutely regulated by Na^+, K^+ -ATPase activity [5] and phosphate and $1,25(\text{OH})_2\text{D}$ levels are suppressed by FGF23 signals at late phase [8]. In this system, FGF23 signals lead to the suppression of *α -klotho* gene expression and the decreased synthesis of $1,25(\text{OH})_2\text{D}$, an enhancer of *α -klotho* gene expression. A negative correlation between sKl and FGF23 levels could reflect this putative feedback system.

FGF23 concentrations are maintained at a low or normal level regardless of high phosphate concentrations in children (Fig.S1). Since high phosphate has been reported to induce high FGF23, low or normal FGF23 concentrations in children seem to be paradoxical. Although the mechanism is unknown, this is postulated to be related to a higher need for adequate bone mineralization during childhood. In contrast, sKl is maintained at a high concentration in children. Based on the preceding statistics that sKl correlates positively with phosphate, the high value of sKl is not inconsistent with a high phosphate level even at a young age. The balance of mineral metabolism in children is different from that in

adults. It is very likely that a shift of metabolic equilibrium sustains higher phosphate concentration and optimizes Ca^{2+} reabsorption in tubules. Further studies are required to delineate the mechanism.

The manifestations seen in the patient with the *α -klotho* gene mutation resembled features observed in *α -klotho* ablated mice, although the patient's phenotype was milder than that observed in the mouse model. In an attempt to understand the manifestations seen in this patient, we verified sKl levels using the ELISA system. Since the mutated protein impairs the intracellular trafficking ability in vitro, the sKl level of the patient was expected to be low. Indeed, the sKl value was more than 50% lower than the mean value of healthy age-matched subjects. Accordingly, the ELISA may be useful in studying disorders of mineral metabolism.

sKl levels are higher in adolescence and lower in older adults and negatively correlate with Cre and BUN, which are increased in an age-dependent fashion even in healthy humans. Therefore, sKl concentrations in serum are expected to be noted as a biomarker for kidney function. In the present study, we are not able to analyze the effect of renal function on sKl and vice versa, since our subjects had normal renal function. Notably, in renal failure, *α -klotho* mRNA expression is decreased [18], though this may be due to death of the cells in the nephron expressing α Kl. Further study is necessary to determine the utility of this ELISA in renal failure.

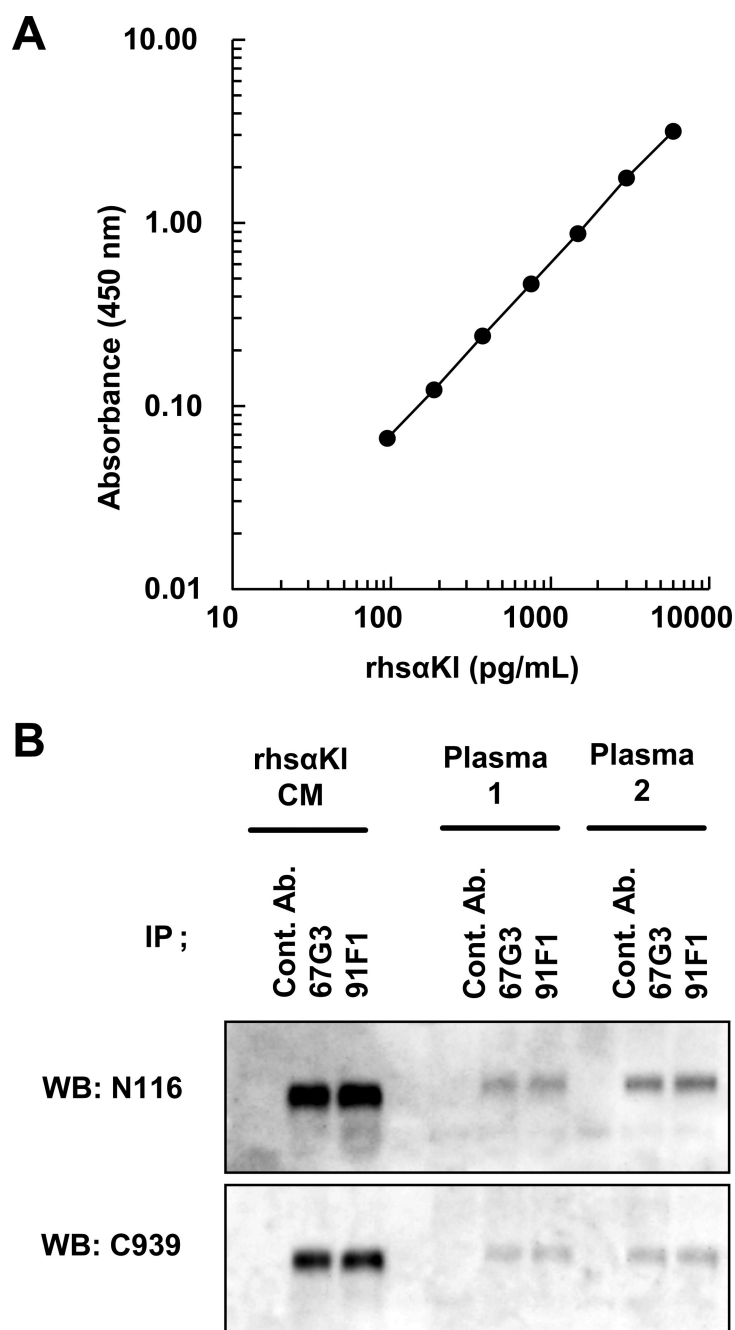
Kurosu et al reported that an increase of sKl levels by genetic manipulation of full-length α -Klotho resulted in a prolonged life span in mice [19]. In that paper, since the levels of normal sKl in mice were measured at >10ng/ml by radioimmunoassay [19], they were estimated to be approximately 20 fold greater than the average normal human sKl concentration in this study. However, as previously reported, murine sKl levels were calculated to be similar to that of humans by immunoprecipitation followed by western blot analysis [6]. These findings indicate that serum sKl concentration should be carefully estimated to evaluate the roles of sKl level in life span elongation. In addition, whereas two to three-fold elevations in sKl level were noted in longevity-elongated mice [19], this magnitude would be within the divergence seen in healthy human subjects. Furthermore, this cross-sectioned study identified that younger subjects have higher sKl, and that older subjects (who have lived longer) have lower sKl. Therefore, careful prospective longitudinal studies would be needed to determine whether future longevity can be predictable from sKl concentrations at one point in time.

Acknowledgments

This work was supported in part by the grants from Ministry of Education, Science and Culture in Japan, 21026017, 21390058 (to A.I.) and 17109004 (to Y.N.) and the Ministry of Health, Labour and Welfare of Japan KH20Q007a-1 (to K.O.). We thank K Sakuma, K Ono and N Yoshii for excellent technical support and Dr. T Sakai for helpful suggestion. We thank the coordinators of Fujiwara-kyo Study [20] from which almost healthy adults were recruited. Notably, subjects from Fujiwara-kyo Study had no history of cardiovascular diseases and diabetes mellitus and no other known medical conditions. Their blood pressures were within the normal range and their serum creatinine levels were less than 1.2mg/dL.

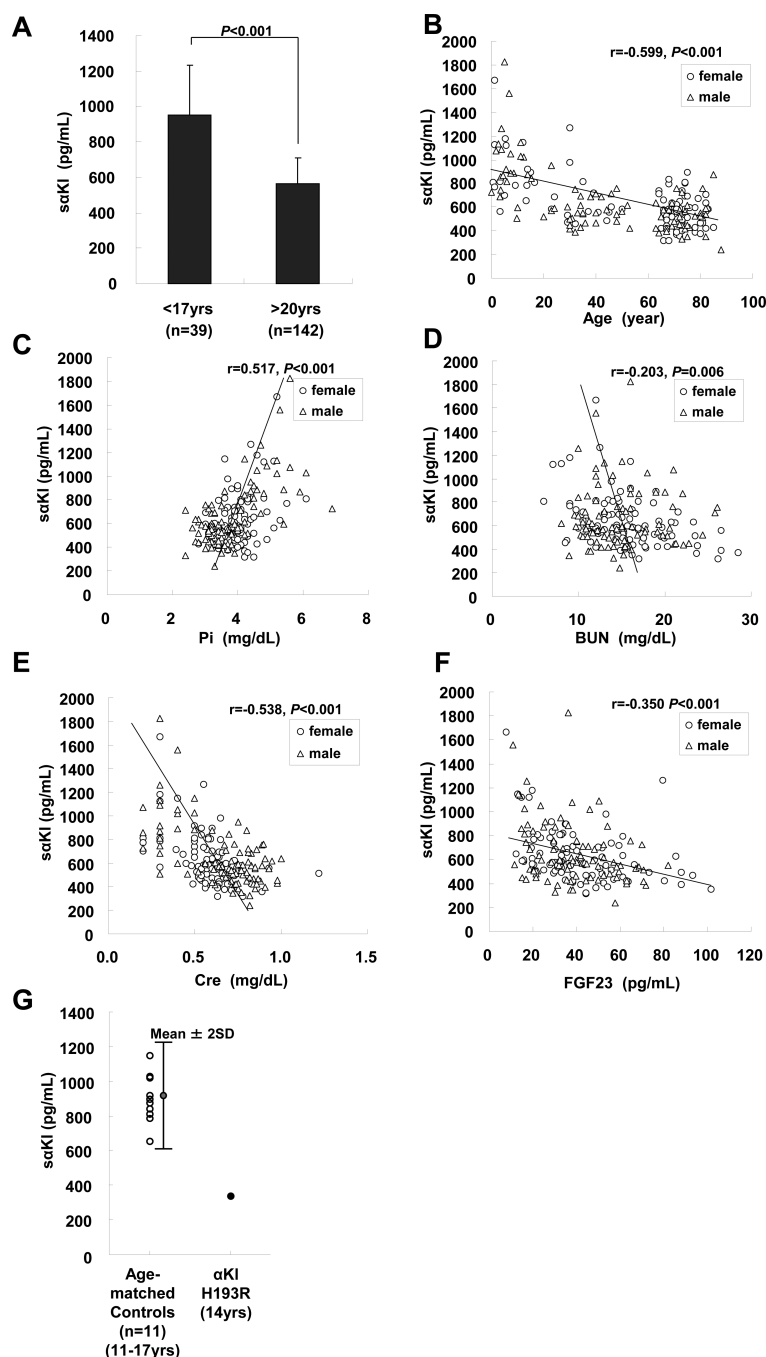
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**Fig.1.**

Enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation analysis by two useful anti-sKl monoclonal antibodies. (A) Typical dose-response curve of recombinant human sKl (rhsKl). Sandwich ELISA for sKl was performed by 67G3 as a capture antibody and 91F1 as a detection antibody. The results are expressed as the mean \pm standard deviation (SD) of triplicate measurements. (B) Immunoprecipitation of plasma sKl by 67G3 and 91F1 antibodies. Conditioned medium from CHO cells stably expressing FGF23 (rhsKl) and plasma from healthy volunteers (plasma 1, plasma 2) were immunoprecipitated

anti-Klotho antibodies (67G3 and 91F1) and control mouse IgG1 (cont. Ab.), and analyzed by Western blot with anti-human Klotho rabbit polyclonal antibodies, N116 and C939.

**Fig.2.**

Serum sKl levels (A) Comparison of serum sKl levels between healthy adults and children. Serum sKl levels of healthy adults (n=142, age: >20 years) and children (n=39, age: <17years) were measured. Results represent mean \pm standard deviation (SD). Statistical analysis was performed by student's t-test. P values less than 0.05 were taken as statistically significant. Correlation of serum sKl with (B) age (C) Pi (D) BUN (E) Cre and (F) FGF23 levels in simple regression analysis in healthy subjects. The open circles and triangles represent male and female subjects, respectively. Statistical analysis was performed by

Pearson correlation. P values less than 0.05 were taken as statistically significant. (G) Comparison of serum sKl levels between a patient with klotho missense mutation and age-matched healthy subjects. Serum sKl levels from 11 healthy subjects (left, age: from 11.2 to 16.6 years) and a patient with klotho homozygous gene mutation (right, age: 14 years) were shown. An error bar along the values for healthy controls represents mean \pm 2 fold of standard deviation (2SD) in order to indicate a reference range.

Table 1

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number:	total (male/female)	all 181 (89/92)	children (<17yrs) 39 (23/16)	adults (>20yrs) 142 (66/76)	<i>P</i> children vs adults
Age	year	49.4 ± 27.7	7.1 ± 4.8	61.1 ± 18.5	<0.001
Ca	mg/dL	9.50 ± 0.44	9.65 ± 0.52	9.46 ± 0.41	0.018
Pi	mg/dL	3.90 ± 0.75	4.88 ± 0.68	3.63 ± 0.51	<0.001
Ca x Pi	(mg/dL) ²	37.1 ± 8.0	47.2 ± 7.9	34.4 ± 5.3	<0.001
BUN	mg/dL	15.2 ± 4.3	13.3 ± 3.6	15.7 ± 4.3	0.002
Cre	mg/dL	0.62 ± 0.19	0.36 ± 0.12	0.69 ± 0.14	<0.001
Alb	mg/dL	4315 ± 384	4190 ± 474	4349 ± 349	0.021
1,25(OH) ₂ D	pg/mL	ND	ND	54.8 ± 17.7	ND
iPTH	pg/mL	ND	ND	49.2 ± 20.6	ND
calcitonin	pg/mL	ND	ND	26.0 ± 12.3	ND
FGF23	pg/mL	39.6 ± 18.4	24.2 ± 12.0	43.8 ± 17.6	<0.001

ND: not determined